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Assessing the effect of high doses of ampicillin on five marine and freshwater phytoplankton species: a biodegradation perspective

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Abstract

Previous studies have identified several effects of antibiotic exposure at doses typically found in natural water (~μg). However, high doses of antibiotics can be found near wastewater treatment plants, and antibiotic concentrations in natural watercourses are likely to increase due to continuous current inputs. Therefore, the systematic evaluation of the susceptibility of phytoplankton species to antibiotics in water should be conducted for an improved risk assessment and the development of biotechnology for antibiotic residue management. The aim of the present study was, therefore, to investigate the response to high concentrations of ampicillin in several microalgae and cyanobacteria species with consideration of potential biodegradation applications for industrial and sanitary wastewaters. Pure laboratory cultures of freshwater (*Dictyosphaerium chlorelloides*, *Chlamydomonas reinhardtii* and *Microcystis aeruginosa*) and marine (*Emiliania huxleyi* and *Prochloron* sp.) species were exposed to several doses of ampicillin (6–14 mg L^{-1}). Cell growth and other functions were followed in each species for up to a month. The results revealed that the species susceptibility to ampicillin varied greatly. No effect was observed in the chlorophytes, *M. aeruginosa* presented high inhibition and microcystin stimulation, upregulation/enhancement occurred in *E. huxleyi*, and photochemical stress occurred in the marine cyanobacterium *Prochloron* sp. Moreover, we observed that the ampicillin effect varied over time in susceptible species. Despite the variability of response, all the species presented high rates of antibiotic degradation. From these bioassays, it can be inferred that the effect of ampicillin cannot be generalized to microalgae groups. Additionally, the potential of microalgae to mitigate antibiotic impacts by degradation is a novel aspect yet to be investigated.

Keywords Microalgae · Cyanobacteria · Ampicillin · Biodegradation · Antibiotics · Species sensitivity

Introduction

Since the revolutionary discovery of antibiotics, the overall level of consumption of these medications has continuously risen (Van Boeckel et al. 2014). "Wonder drugs" remain essential to reduce the burden of infectious diseases. Medical necessity notwithstanding, over the past decades, there has been growing concern about the undesirable effects of antimicrobial agents, mainly related to environmental impacts (Ding and He 2010; Grenni et al. 2018) and escalating antibiotic resistance (Knapp et al. 2010). As a result of broad utilization in human and veterinary medicine, in livestock husbandry and aquaculture, as growth promoters (practice withdrawal in the European Union (EU Regulation 1831/2003)) and in production processes, substantial amounts of antibiotics are released into the ecosystems (Quezada et al. 2012). Consequently, the main source of antibiotic release in the environment proceeds from sewage waters from urban, industrial, and animal husbandry sources that are not degraded during wastewater treatment (Christian et al. 2003; Dolliver et al. 2008; Kemper et al. 2008; Li et al. 2008). However, antibiotic manufacturing facilities have been reported to be sources of much higher environmental concentrations compared with those related to the consumption of antibiotics (Larsson et al. 2007; González-Plaza et al. 2018). Therefore, antibiotics, even those

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considered highly degradable, have been detected throughout the world, especially in wastewater-impacted surface and ground waters (Kasprzyk-Hordern et al. 2008; Snyder et al. 2009; Watkinson et al. 2009), ultimately reaching seawater (Ali et al. 2017), due to continuous inputs. Antibiotics are recognized as emerging compounds in the water environment whose effects, both for public and ecological health, are still not fully understood.

Increasing awareness of aquatic environment degradation has led to a growing investigation related to the impact of antibiotic agents on nontarget microbial natural communities (Costanzo et al. 2005; Baquero et al. 2008; Kümmerer 2009a, b; Martinez 2009; González-Pleiter et al. 2013; Zhang et al. 2013; Grenni et al. 2018). Phytoplankton represent the foundation of aquatic environments, constituted by a polyphyletic group of autotrophic microorganisms, both prokaryotes and eukaryotes (Simon et al. 2009). Species within the phytoplankton are responsible for approximately half of the primary production on earth (Field et al. 1998; Schiermeier 2010; Falkowski and Raven 2013) and determine the abundance and geographical distribution of the aquatic biome. Environmental factors are determinant in phytoplankton distribution and abundance and are consequently affected by human-induced global changes (Behrenfeld et al. 2006; Boyce et al. 2010; Forest et al. 2011). Previous studies have shown that phytoplankton can be potentially hindered by antimicrobial substances (Halling-Sorensen 2000; Lopez-Rodas et al. 2001; Costanzo et al. 2005; Campa-Córdova et al. 2006; Lai et al. 2009; Kümmerer 2009a; van der Grinten et al. 2010; Liu et al. 2012a; González-Pleiter et al. 2013). For example, antibiotics have a direct biological effect on phytoplankton community structure, especially broad-spectrum antibiotics that act upon different groups of microorganisms, and effects on phytoplankton natural functions, P fixation, and N cycles have also been discovered. The number of studies focusing on the effect of antibiotics in phytoplankton species is continuously rising with the aim of bridging the knowledge gaps (i.e., combined effects of antibiotic mixtures (González-Pleiter et al. 2013; Liu et al. 2014)). Regarding the current increasing trend of antibiotic consumption and discharges, addressing the susceptibility to antibiotics at concentrations far above environmentally relevant concentrations can offer insights into extreme scenarios of antibiotic pollution and possible bioremediation practices.

The most common class of antibiotics are broad-spectrum ß-lactams (Kümmerer 2009a), and among this class of antibiotics, one that has a strong trend of consumption and has been used for a long time is ampicillin (AMP) (De Magalhaes and Borschiver 2012; Versporten 2013). AMP was the first broad-spectrum penicillin and has been extensively used since the 1960s (Acred et al. 1962). Although concentrations of AMP residues in water are usually low at ng L^{-1} levels (Kümmerer 2001; Fatta-Kassinos et al. 2011) and are efficiently removed during wastewater treatment (Li and Zhang 2010), AMP is reported from concentrations of several μg L^{-1} in effluents of wastewater treatment plants (WWTPs) (Christian et al. 2003; Kulkarni et al. 2017) to mg L^{-1} in wastewaters from pharmaceutical production (Jun and Zhengyu 1997; Zhou et al. 2006). Moreover, not all products of AMP hydrolysis are screened, some could act as active compounds, and most substances do not have a regulatory status (Mitchell et al. 2014). Therefore, AMP and its hydrolysis products are probably underestimated in waterways. In terms of action, AMP inhibits the enzyme transpeptidase and therefore bacterial cell wall synthesis. Due to its mode of action, AMP has been widely used to obtain and maintain axenic algae cultures, but only a few studies have assessed the effects of AMP on microalgae and cyanobacteria cultures (Kvíderová and Henley 2005; Liu et al. 2012a; Dias et al. 2015). A further understanding of the impacts of AMP on microorganisms of the aquatic biome is important for risk assessment and management.

In the present study, we aimed to evaluate the susceptibility to AMP of different phytoplankton species, including eukaryotes and cyanobacteria, from marine and freshwater environments, different from the species recommended in the OECD 201 guideline (OECD 2011). The growth rates, photosynthetic activity, and microcystin (MC) production of *Microcystis aeruginosa*, and the degradative rate of AMP were investigated. The tested exposure doses ranged from mg L^{-1}, and the effect was studied for a month after the exposure, addressing worst-case scenarios of AMP pollution, such as sanitary and industrial WWTPs pollution. Our results will contribute to wider knowledge related to the environmental risk of a guided-use broad-spectrum antibiotic such as AMP for phytoplankton populations. The eventual goal of this work is to provide insights for the development of microalgae-mediated biotechnology and bioremediation methods for antibiotics, especially AMP.

Materials and methods

AMP stability assessment

The initial concentrations of the stock media and AMP stability were examined. The stability of 10 mg AMP L^{-1}, the intermediate concentration of those tested, was analyzed in exposure broths without cells and in each study strain during the time course of the bioassay for up to 28 days. The pH variations from the initial circumneutral values were monitored in the bioassays. The pH was measured with a pH meter (Hach session + PH3, USA).

The analysis of AMP in the biological samples was conducted by high-performance liquid chromatography (HPLC) using a previously validated method based on the

methodology proposed by Injac et al. (2009), modified and adapted to our study. Briefly, a modular HPLC (Jasco International, Japan) equipped with an LG-2080-04 quaternary low-gradient unit, a PU-2080 pump, a DG-2080-54 degasser, an AS-2050-plus autosampler, and a UV-2070 plus UV/Vis detector was used. A C18 column (Kromasil 100 4.6 mm × 250 mm, 5-μm particle size; Scharlab, Spain) was selected for the chromatographic separation of the components, with a column temperature of 30 °C (LC Ni-II/ADC column oven). The composition of the mobile phase was a mixture of MilliQ water and acetonitrile 89:10 (*v*/*v*). Solvents were vacuum-filtered through 0.45-μm nylon Millipore membranes (Millipore, USA) and degassed by ultrasonication for 20 min before use. The flow rate was set at 1.0 mL min^{-1}, and the injection volume was 100 μL. After obtaining a stable baseline, aliquots of samples were injected. AMP exhibited a retention time of approximately 5 min. The total run time was set to 15 min to cover all possible impurities present within the biological samples, as previously checked. Standard references were introduced for analyses every five study samples. The areas of the chromatographic peaks were monitored at 210 nm and compared with standard samples of ampicillin in the linear range between 0.1 to 20 mg L^{-1} (SD1). To exclude possible interferences or false positive results, culture media and ancestral cultures of the five strains studied were analyzed to ensure the absence of AMP (data not shown).

Samples were centrifuged (15,000 *g* for 15 min) to eliminate the cells. Each aliquot analyzed consisted of 150 μL at a 1:10 dilution with type I (ultrapure) water filtered using a 0.45-μm PVDF syringe filter (Millipore Millex-HV).

Microorganisms and culture conditions

Five different phytoplankton strains from the culture collection of Universidad Complutense de Madrid (Madrid, Spain) were used. Three of them originated from pristine freshwater areas: two were isolated from Doñana National Park (southern Spain), *Microcystis aeruginosa* (Kützing) Lemmermann (Cyanobacteria) (toxic strain Ma12D) and *Chlamydomonas reinhardtii* Dangeard (Chlorophyta) (strain Cr1D), and *Dictyosphaerium chlorelloides* (Nauman) Komárek and Perman (Chlorophyta) (strain Dc1M) was isolated from Sierra Nevada National Park (Southern Spain). The other two strains were isolated from oceanic waters: *Emiliania huxleyi* (Lohmann) Hay et Mohler (Haptophyta) (strain Eh1S) was isolated from the Sargasso Sea originally from UTEX stock, and *Prochloron* sp. Lewin ex Hoffman and Greuter (Cyanobacteria) (strain Pc1C) was isolated from the Gulf of Cadiz (Spain). Each isolate stemmed from a single cell obtained using a micromanipulator (Zeiss Eppendorf) and reproduced asexually, originating in a clonal strain. The original clonal isolates were propagated and transferred for more than a year. Populations were not recloned before the experiments to simulate more natural conditions, with higher genetic variability within the populations.

Cultures were maintained in filter-cap cell culture flasks (Greiner, Bio-one Inc., USA) with 20 mL of BG-11 medium and F/2 medium (Sigma-Aldrich, Germany) for freshwater and marine strains, respectively. F/2 medium was prepared with filtered seawater (0.22 μm, Millipore Steritop, Fisher Scientific) collected from the Mediterranean Sea at the Gulf of Cadiz, Spain. The strains were maintained axenically at 20 ± 2 °C under continuous illumination provided by white fluorescent tubes at 70–80 μmol photons m^{-2} s^{-1} over the wavelength between 400 and 700 nm and in an exponential mid-log growth by serial transfers to fresh medium every 20 days.

The study antibiotic was ampicillin (AMP) Sigma-Aldrich. Stock solutions were prepared immediately before the start of the experiments by direct dilution of the antibiotic powder in freshwater or marine media, and the pH values were adjusted to 7 and 8, respectively, if necessary. The initial concentrations of the exposure solutions were measured before the experiment. The stability of the AMP in the exposure solutions, subjected to the same culture conditions, was monitored along with each independent experiment.

Long-term bioassays for the susceptibility to AMP

Five independent AMP susceptibility assays were performed, one for each of the five different microalgae and cyanobacteria study strains. Eighteen replicate populations of each strain were established in 14 mL CELLSTAR transparent cell culture tubes with a two-position vent stopper, with a total volume of 9 mL of freshly prepared exposure solution and an initial population of 10^6 cells under culture conditions (20 ± 2 °C and continuous illumination). In groups of three, replicates of each strain were stabilized in control media without AMP and in exposure solutions of 6, 8, 10, 12, and 14 mg L^{-1} AMP. The lowest test concentration was two times the maximal AMP concentration described in pharmaceutical WWTPs (3.2 mg L^{-1} AMP (Zhou et al. 2006)), and the concentration was increased by 2 mg L^{-1} up to the highest concentration studied to be comparable with previous biodegradation studies (Shen et al. 2010). These concentrations were several fold higher than those previously detected to address worst-case pollution scenarios.

A test with a range of exposure times was performed to assess the effects of different AMP concentrations on each species, assessing the long-term response of the populations to acute exposure. During each independent bioassay, one tube with a stock solution was maintained under experimental conditions. Every 3 days all tubes were rotated along with the tube holder one position to the right to reduce spatial position effects. The tubes were maintained under culture conditions without transfers up to 28 days.

Measurements were made at the beginning and every 7 days ($t = 7$ days, $t = 14$ days, $t = 21$ days, and $t = 28$ days) by subtracting a volume of 1 mL from each sample. Independent experiments were conducted for 3 consecutive months.

Specific growth rates were calculated by means of the Malthusian component of fitness (m) with the following equation:

$$N_t = N_0 e^{\mathrm{mt}} \text{ (Crow\&Kimura 1970)}$$

where t = time in days and N_0 and N_t are the cell densities at the beginning and at t.

The toxic effect of AMP was quantified based on the % inhibition (% I).

$$\%\text{inhibition} = 100\left(1-\frac{m_{\text{treatment}}}{m_{\text{control}}}\right)$$

Cell counts in experimental samples and controls were made using an inverted compound microscope and disposable counting grids chambers (FastRead 102, Fisher Scientific, France) at different magnifications. Three complete grids were counted in each replicate population; the mean of which was used to estimate the population densities.

Photosynthetic chlorophyll *a* fluorescence activity measurements

The photosynthetic response to AMP exposure was evaluated by fluorometry with an Imaging-PAM chlorophyll fluorometer (Maxi version, Walz, Germany). PAM fluorimeters shed pulse-modulated light to excite the fluorescence of chlorophyll *a*. The Imaging-PAM enables measurements in algal suspensions by analyzing images of fluorescence emission in the selected sample area (areas of interest). Specifications of the Imaging-PAM device were described in depth by Ralph et al. (2005).

Minimum fluorescence state (F_0) and variable fluorescence (F_v/F_m) were measured in flat-bottomed 48-well plates with 0.5 mL of the parental population at the initial density (data not shown) and 0.5 mL of each replicate of the AMP bioassay every 7 days. Cells densities were blindly counted before the quantifications. Samples were adapted to darkness 30 min before the measurements to obtain the optimal quantum yield (F_v/F_m) of photosystem (PS) II. Variable fluorescence (F_v/F_m) illustrates PS II activity, providing an estimation of the well-being of the cell (Ohad et al. 2010). All measurements were carried out at 22 °C, where PS I emits weak fluorescence and cannot be clearly distinguished from the main PS II fluorescence (Itoh and Sugiura 2004). Images of the dark-adapted state were recorded; first, an image of the dark-adapted minimum fluorescence state (F_0) was recorded before the application of an excitation pulse (2700 μmol photons m^{-2} s^{-1} provided by the blue LED ring at 470 nm) providing the dark-adapted maximum fluorescence state (F_m). From F_0 and F_m, the optimal PS II quantum yield (F_v/F_m) can be calculated: $F_v/F_m = (F_m - F_0)/F_m$.

The recorded digital images were analyzed by analytical software (Imaging-WIN, Walz) to obtain the numerical values of the chlorophyll *a* fluorescence parameters.

Measurements were performed for the two Chlorophyta (Cr1D and Dc1M) and the marine cyanobacterium *Prochloron* sp. (Pc1C) strains. The fluorescence parameters of the *M. aeruginosa* and *E. huxleyi* strains presented low repeatability, and the measured error was very high, even in high-density cultures; therefore, the results were not reliable. These issues could be related to the device specifications or the low chlorophyll content of the strains.

Microcystin production evaluation

The extracellular microcystin-LR equiv. (MC-LR) production of the toxic strain Ma12D of *M. aeruginosa* was measured during the AMP exposure experiment in the control replicates and the maximum AMP dose (14 mg L^{-1}) replicates. MC-LR is the most frequently detected MC variant produced by *M. aeruginosa* (Dietrich and Hoeger 2005) and was selected as a target chemical.

The parental population was assessed at the late logarithmic growth phase when the MC-LR concentration was higher (Watanabe et al. 1996; Vezie et al. 1998). Aliquots from one randomly chosen replicate of each group, controls and 14 mg L^{-1} AMP replicates, were kept at the four sampling times to address the evolution of microcystin production. The cell concentration was assessed in each replicate prior to the determination.

Quantification of the toxin production of the Ma12D replicates was performed with the commercial MicroCystest Kit (ZEU-INMUNOTEC, Spain). This kit is a quantitative test kit approved by the Environmental Technology Verification program of the US Environment Protection Agency (EPA). It detects potentially toxic microcystin residues by means of the inhibition of protein phosphatase 2A (PP2A). The working range is 0.25–2.5 μg L^{-1}, and the detection limit is 0.08 μg L^{-1}. Prior to the measurement, each aliquot was filtered through Millipore syringe filters (0.22 μM) to eliminate the cells (Merck, Germany). Following the manufacturer's guidelines (Smienk et al. 2007), absorbance measurements of the three standards (with known concentrations of 0.5, 1, and 2.5 μg L^{-1} of MC-LR) and the study samples were recorded at 405 nm. The results of microcystin evaluation

were estimated from the equation of a logarithmic trend line best-fit curved line and expressed as μg L^{-1}.

Statistical analyses

All statistical analyses were conducted with the software package R (R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org). Throughout this paper, the data were displayed as the mean of three replicates and the standard deviation, unless otherwise stated. Significance levels were considered at $P < 0.05$. After the data were tested for normality and homogeneity of variance of the linear model, one-way ANOVA and post hoc Tukey's pairwise comparisons were used to test for significant differences in the number of cells and bioassay growth rates per sampling day. Fluorescence values were analyzed with nonparametric tests comparing fluorescence values per day. MC production by the Ma12D strain was a function of the following main effects: the age of the culture in days and the treatment. Two-way analysis of covariance (ANCOVA) was used for continuous, normally distributed effects of AMP exposure or lack of exposure on MC-LR production, with treatment (control and 14 mg AMP L^{-1}) as a factor and age of the culture as a continuous covariate. Comparisons between the parental population and the bioassay control MC values at 28 days were conducted using Student's *t* test.

Results

Quality control of AMP stability

No significant differences were found between the nominal AMP concentration and the measured concentration at the beginning of the experiment or between the broth media (abiotic conditions) AMP concentrations at each sampling time. AMP stability in broth media (abiotic conditions) was measured under bioassay conditions resulting in degradation, expressed as a percentage of the measured initial concentration of 89.5 ± 1.79, 84.25 ± 4.48, 75.76 ± 3.45, and 74.28 ± 0.93 of the average concentrations ± s.d. after 7, 14, 21, and 28 days, respectively (Fig. 1). The results of Fig. 1 demonstrate that the stability of AMP in the biological assays was significantly reduced at day 7, reaching values close to 0 at day 14, except in the marine cyanobacteria Pc1C, where the AMP dropped to approximately 10% of the initial concentration and remained at that level until day 28. Both Chlorophyta biological assays presented increased values of AMP at days 21 and 28, and effects were more significant in Cr1D.

The pH ranged from slightly acidic to alkaline at the end of the bioassay for all the strains. For Chlorophytes Dc1M and Cr1D, the increase in the pH was within 2–2.5 units, and for the freshwater cyanobacteria, it did not exceed 1.5–2 pH units. For the two marine strains Eh1S and Pc1C, the pH values were in the range of 7.9–8.3 and 7.8–8.5, respectively.

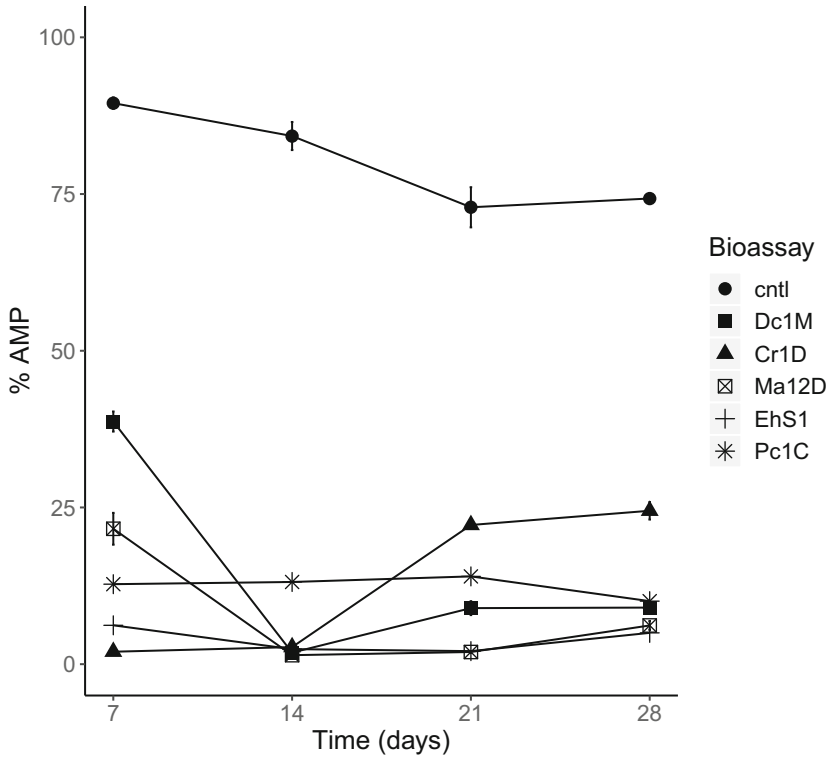

Fig. 1 AMP concentration as a percentage of the measured initial concentration over time in the stock media (cntl) and per strain: *D. chlorelloides* (Dc1M), *C. reinhardtii* (Cr1D), *M. aeruginosa* (Ma12D), *E. huxleyi* (Eh1S), and *Prochloron* sp. (Pc1C). Error bars represent one standard deviation

AMP susceptibility of five microalgae strains

Dose-response relationships

Our results indicate that AMP susceptibility depends primarily on the phytoplankton strains studied (representing five different species of marine and freshwater cyanobacteria and microalgae). In response to AMP exposure, in general, three different patterns were observed among test strains: (a) those not particularly susceptible to AMP, (b) those negatively affected, and (c) those positively influence with an increase in cellular proliferation or improved yield. In addition, the AMP effects were also dependent on the time of exposure/age of the culture and the antibiotic concentration.

With respect to the AMP effect on cellular growth, the dose-response curves of each strain exposed for 28 days are depicted in Fig. 2a–e for each study strain. The results are represented by the number of cells expressed as the mean ± s.d. of three independent replicates per treatment. Among the five strains studied, as exemplified from Fig. 2, only *M. aeruginosa* (Ma12D) presented a strong reduction of cell density during the experiment at the lowest AMP concentration (linear model one-way ANOVA significant differences from the control at all AMP treatments and sampling times,

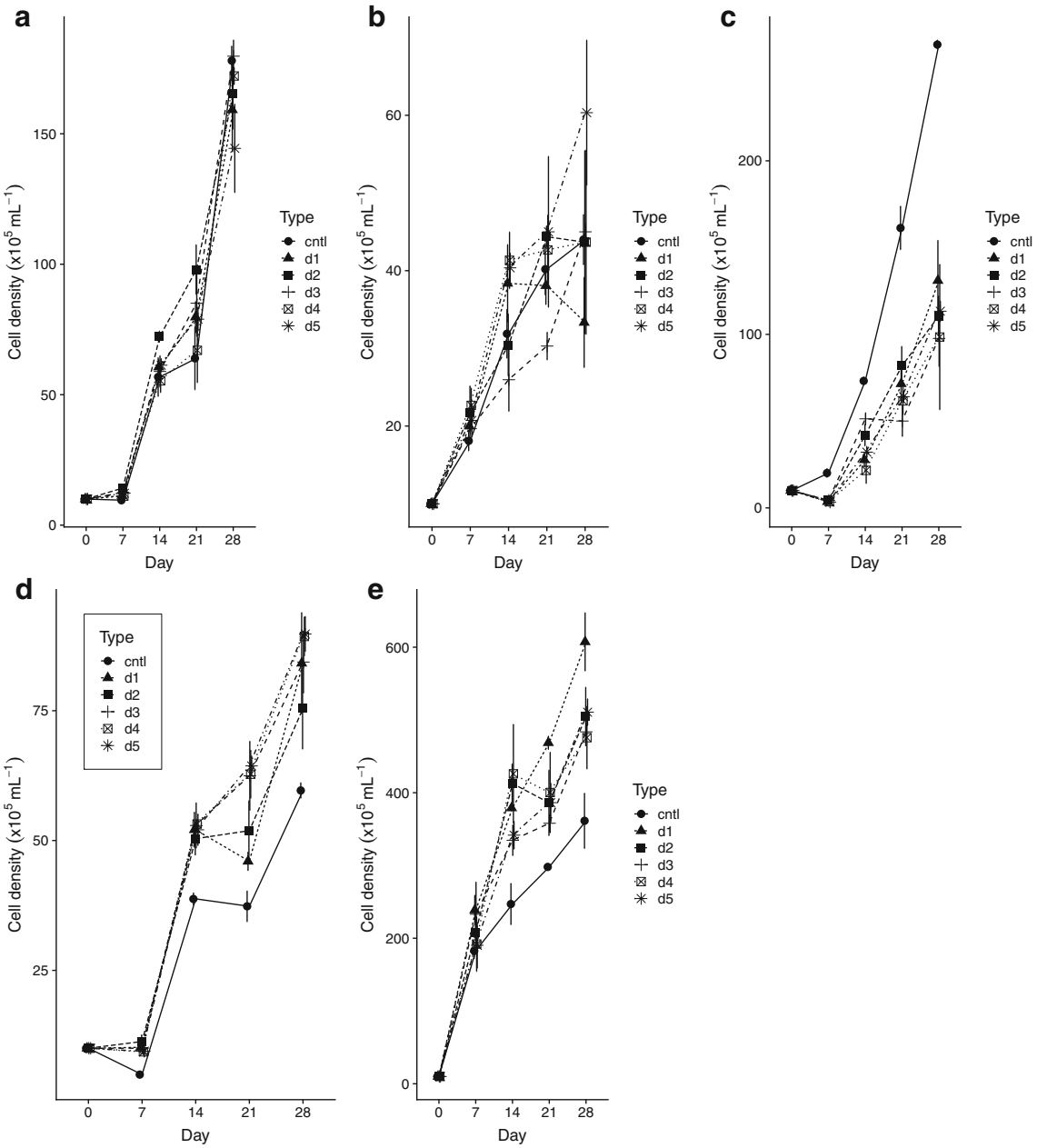

Fig. 2 Growth curves of the five strains of study at different AMP concentrations over time. Means ± SE from three separate experiments are shown. **a** *D. chlorelloides* (Dc1M). **b** *C. reinhardtii* (Cr1D). **c** *M. aeruginosa* (Ma12D). **d** *E. huxleyi* (Eh1S). **e** *Prochloron* sp. (Pc1C)

$P < 0.05$, (ST1)). A pronounced inhibition of Ma12D growth was observed over the experiment; consistently, the % inhibition expressed according to the control group ranged between 80.839 ± 2.426% at day 7 and 45.129 ± 7.760% at day 28 (ST1). Growth inhibition at the different AMP concentrations of the Ma12D strain was not significantly different, presumably because the effect was assessed after 7 days of exposure at high AMP doses and the inhibition values were higher than 80%. The two Chlorophyta strains, *D. chlorelloides* (Dc1C) and *C. reinhardtii* (Cr1D), showed normal growth during the experiments and were not susceptible to any AMP concentration (6–14 mg L^{-1}) throughout the exposure time (c7.43 and c5.42 generations, respectively, Fig. 2a, b). Alternatively, both marine strains, *E. huxleyi* (Eh1S) and *Prochloron* sp. (Pc1C), exhibited higher average cell densities under AMP exposure treatments (Fig. 2d, e).

Similar patterns can be observed in the estimated growth rate (r) values of the five strains (Table 1), but in some cases r comparisons were required to better elicit the AMP treatment effect. The r values of Ma12D were severely affected by all concentrations studied with respect to the control at days 7 and 21, but at 28 days, no significant differences in the r values were observed. None of the Chlorophyta strain r values were affected by AMP exposure during the experiment. In the Eh1S strain, more growth was observed when exposed to all AMP concentrations than when incubated without antibiotics for the first 14 days and this prevailed during the experiment for higher AMP concentrations, that is, 10–14 mg L^{-1} at 21 days

Table 1 Growth rate (r) values (expressed as mean ± s.d.) of five microalgae strains: *D. chlorelloides* (Dc1M), *C. reinhardtii* (Cr1D), *M. aeruginosa* (Ma12D), *E. huxleyi* (Eh1S), and *Prochloron* sp. (Pc1C). Italicized letters represent r values significantly different from the control values identified in the course of the bioassays ($P < 0.05$)

Growth rate (r) values

Strain per day	Treatment						Statistics	
	cntl	6 mg L^{-1}	8 mg L^{-1}	10 mg L^{-1}	12 mg L^{-1}	14 mg L^{-1}	F value	P
D. chlorelloides (Dc1M)								
7	0.319 ± 0.036	0.346 ± 0.014	0.378 ± 0.013	0.355 ± 0.048	0.338 ± 0.05	0.357 ± 0.002	1.08	> 0.05
14	0.287 ± 0.017	0.293 ± 0.005	0.305 ± 0.003	0.290 ± 0.014	0.286 ± 0.010	0.293 ± 0.005	1.397	> 0.05
21	0.196 ± 0.015	0.208 ± 0.005	0.217 ± 0.008	0.210 ± 0.012	0.198 ± 0.015	0.207 ± 0.004	1.475	> 0.05
28	0.184 ± 0.001	0.180 ± 0.006	0.182 ± 0.005	0.185 ± 0.002	0.183 ± 0.001	0.177 ± 0.006	1.446	> 0.05
C. reinhardtii (Cr1D)								
7	0.413 ± 0.017	0.429 ± 0.005	0.436 ± 0.037	0.425 ± 0.004	0.444 ± 0.025	0.431 ± 0.025	0.673	> 0.05
14	0.246 ± 0.012	0.259 ± 0.015	0.242 ± 0.016	0.230 ± 0.020	0.265 ± 0.010	0.269 ± 0.006	2.846	> 0.05
21	0.175 ± 0.007	0.173 ± 0.005	0.181 ± 0.002	0.162 ± 0.004	0.178 ± 0.008	0.179 ± 0.017	1.652	> 0.05
28	0.134 ± 0.004	0.124 ± 0.011	0.132 ± 0.017	0.136 ± 0.001	0.132 ± 0.018	0.145 ± 0.010	0.998	> 0.05
M. aeruginosa (Ma12D)								
7	0.426 ± 0.032	*0.177 ± 0.046*[¥]	*0.199 ± 0.68*[¥]	*0.208 ± 0.038*[¥]	*0.178 ± 0.031*[¥]	*0.162 ± 0.067*[¥]	11.97	< 0.001[**]
14	0.306 ± 0.003	*0.235 ± 0.020*[¥]	*0.265 ± 0.017*	*0.281 ± 0.008*	*0.211 ± 0.043*[¥]	*0.247 ± 0.012*[¥]	7.482	< 0.001[**]
21	0.241 ± 0.006	*0.202 ± 0.015*[¥]	*0.208 ± 0.012*[¥]	*0.184 ± 0.016*[¥]	*0.196 ± 0.010*[¥]	*0.198 ± 0.003*[¥]	9.064	< 0.001[**]
28	0.199 ± 0.002	0.173 ± 0.011	0.167 ± 0.007	0.163 ± 0.011	*0.157 ± 0.028*[¥]	0.168 ± 0.003	3.587	> 0.05[*]
Emiliania huxleyi (Eh1S)								
7	0.227 ± 0.018	*0.329 ± 0.027*[¥]	*0.344 ± 0.027*[¥]	*0.327 ± 0.023*[¥]	*0.318 ± 0.014*[¥]	*0.318 ± 0.028*[¥]	7.866	< 0.01[**]
14	0.261 ± 0.003	*0.282 ± 0.008*[¥]	*0.279 ± 0.007*[¥]	*0.282 ± 0.009*[¥]	*0.283 ± 0.002*[¥]	*0.282 ± 0.007*[¥]	4.641	< 0.01[**]
21	0.172 ± 0.006	0.182 ± 0.003	0.187 ± 0.009	*0.196 ± 0.008*[¥]	*0.196 ± 0.005*[¥]	*0.198 ± 0.002*[¥]	7.645	< 0.01[**]
28	0.146 ± 0.001	0.157 ± 0.007	0.154 ± 0.006	0.158 ± 0.002	*0.160 ± 0.002*[¥]	*0.160 ± 0.002*[¥]	4.252	< 0.01[**]
Prochloron sp. (Pc1C)								
7	0.743 ± 0.015	0.781 ± 0.020	0.762 ± 0.013	0.774 ± 0.047	0.746 ± 0.047	0.746 ± 0.040	0.652	> 0.05
14	0.392 ± 0.014	*0.426 ± 0.001*[¥]	0.429 ± 0.008	0.415 ± 0.008	*0.430 ± 0.021*[¥]	0.416 ± 0.007	4.148	< 0.05[*]
21	0.271 ± 0.001	*0.292 ± 0.001*[¥]	0.282 ± 0.009	0.279 ± 0.003	0.284 ± 0.012	0.283 ± 0.006	3.204	< 0.05[*]
28	0.209 ± 0.006	*0.228 ± 0.004*[¥]	*0.222 ± 0.005*[¥]	0.221 ± 0.001	0.219 ± 0.005	*0.223 ± 0.002*[¥]	5.627	< 0.001[***]

*One-way ANOVA significant differences ($P < 0.05$)

[¥] Tukey's post hoc significant difference from control ($P < 0.05$)

and 12–14 mg L^{-1} at the end of the experiment. The *r* values of Pc1C were enhanced from day 14 at different AMP concentrations, with the 6 mg L^{-1} AMP treatment enhancing the *r* through day 28.

Chlorophyll *a* fluorescence kinetics

Modulated PS II chlorophyll fluorescence was measured to assess AMP-induced stress. Dark-adapted maximum PS II efficiency (F_v/F_m) and minimum fluorescence (F_0) values for the three strains studied are shown in Fig. 3. The different AMP concentrations did not significantly affect the chlorophyll fluorescence of Cr1D and Dc strains; F_v/F_m and F_0 parameters did not differ between control (nontreated cells) and treatment populations. The relatively high mean F_v/F_m (0.932 ± 0.001) in the Dc1M strain at day 7 is attributed to anomalously low values of F_0, probably due to the low cell density. In the Pc1C strain, AMP-treated cultures exhibited higher mean F_0 values from day 14, with significant differences between controls and treatments from day 21 (Wilcoxon

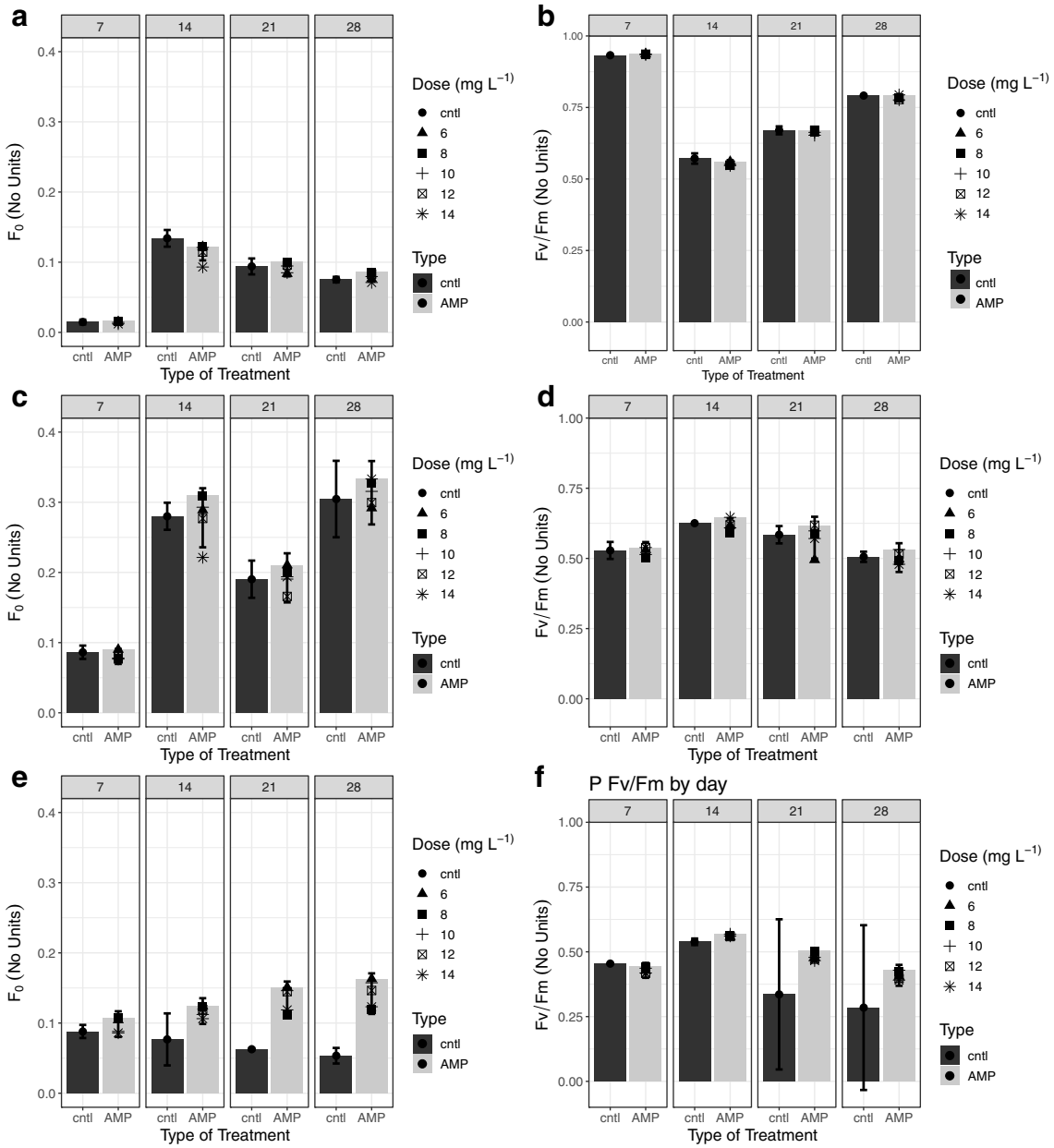

Fig. 3 The values of selected PS II photochemistry parameters measured following dark adaptation in control replicates (cntl) and under AMP at different doses. *D. chlorelloides* F_0 (**a**) and F_v/F_m (**b**) values. *C. reinhardtii* (Cr1D) F_0 (**c**) and F_v/F_m (**d**) values. *Prochloron* sp. (Pc1C) F_0 (**e**) and F_v/F_m (**f**) values. The average values ± s.d. of all the replicates exposed to AMP and the mean values per dose of exposure are shown

rank-sum test, $P < 0.5$). F_v/F_m decreased slightly in the control of Pc1C cultures, although the decrease was not significant and was probably due to the high variability.

Total MC-LR production

The toxin analyses in all the evaluated replicates showed abundant MC-LR concentrations (TS1) according to the equation of the line of best fit ($R^2 = 0.995$). The Ma12D parental population toxin concentration was 1.757 ± 0.008 μg L^{-1} MC-LR at the late logarithmic growth phase, which agrees with the MC-LR values obtained in the control replicates at 28 days ($t = 0.917$, $P > 0.1$). In the toxin production analyses, MC-LR concentrations varied significantly over time and among the different treatments (two-way ANCOVA, time ($F_{3.16} = 431.01$; $P > 0.001$) and treatment ($F_{1.16} = 283.70$; $P > 0.001$) as factors). MC-LR production in the exposure experiment was negatively related to the age of the culture and therefore decreased over time (FS 1). The decrease in MC-LR production may be related to a depletion in the nitrogen supply (Downing et al. 2005; Harke and Gobler 2013; Horst et al. 2014). The regression slopes were negative and different for both treatments, the control and AMP exposure, but the AMP regression line shows intercepts at higher values, indicating an elevated MC-LR production (FS 1).

Discussion

In the present study, we assessed the long-term effect of a single AMP exposure event on five microalgae and cyanobacteria strains. Regarding AMP stability in dissolution, stock solutions under experimental conditions at the endpoint of toxicity presented approximately three-quarters of the initial AMP concentrations. Due to the physicochemical proprieties of AMP (log K_{ow} 1.35 and pKa 2.7–7.3), its hydrolysis half-life is longer than that of other ß-lactam antibiotics. These results fall within the half-lives, 27 days, previously described for AMP at pH 7 and 25 °C (Mitchell et al. 2014). β-lactam hydrolysis is a function of environmental conditions. Thus, we can consider that abiotic factors (photolysis, hydrolysis, etc.) under culture conditions are responsible for 25% of the AMP degradation. However, in the biotic assays, we observed a reduction of up to 90% of the initial AMP concentration at day 14 in all the strains. The apparent increase in AMP concentration after day 14 in *C. reinhardtii* and *D. chlorelloides* could be the result of interference with AMP degradation products, but we have not performed any further analyses. Abiotic degradation of AMP was measured in the stock solutions; therefore, the AMP reduction in the bioassays could be due to several biotic factors (i.e., uptake, biodegradation, or alkalization due to photosynthetic activity). The pH of the exposure medium for all the bioassays varied over the study period from the initial circumneutral in freshwater species media and pH 8 in marine strain media to pH 8.5–9 in *M. aeruginosa* medium, pH 9 to 9.5 in Chlorophyta strain media, and pH 8.3 to 8.5 in marine strain media. Previous studies documented that hydrolysis of AMP was independent of pH at pH values of 4 to 8 (Mitchell et al. 2014). Consequently, the rise in pH linked to microalgae growth might catalyze AMP hydrolysis but does not correlate with the significant AMP degradation displayed in the five strains at day 7. Previous researchers have demonstrated the antibiotic removal efficiency of several algae species (Liu et al. 2012a; de Godos et al. 2012; Yu et al. 2017); the losses of AMP in our study were therefore likely related to the activity of the cells.

AMP exposure induced different patterns of response (no effect, increased values, or inhibition) regarding the biological parameters evaluated. The two Chlorophyta species, *C. reinhardtii* (Cr1D) and *D. chlorelloides* (Dc1M), were not significantly affected by AMP. The two strains showed no sensitivity; cellular growth and PS II photochemistry did not differ from the control values at any dose during the study time. Previous studies on other species of Chlorophyte microalgae and eukaryotic microorganisms showed minor or no effect for most of the individually tested antibiotics, including AMP at high concentrations (several mg L^{-1}) (Holten Lützhøft et al. 1999; Halling-Sorensen 2000; Kvíderová and Henley 2005), except for erythromycin (Eguchi et al. 2004; Isidori et al. 2005; Nie et al. 2013) and some antibiotic mixtures (González-Pleiter et al. 2013). The AMP tolerance displayed by most green microalgae has been attributed to the lack of a peptidoglycan layer in the cell walls. Therefore, AMP combined with other antibiotics is commonly used to obtain axenic microalgae cultures (Kvíderová and Henley 2005). Together with other studies conducted in other Chlorophyte species, these studies show that AMP is suitable for the maintenance of photosynthetic eukaryote cultures for physiological studies. Both marine strains, the cyanobacteria P*rochloron* sp. (Pc1C) and the coccolithophore *E. huxleyi* (Eh1S) showed tolerance to higher concentrations of AMP. Interestingly, over the time course of the *E. huxleyi* bioassay, the replicates exposed to the higher AMP dose displayed better growth performance, suggesting not only resistance to AMP but also a favorable effect of the antibiotic. To our knowledge, AMP at mg L^{-1} has been used to obtain axenic cultures of *E. huxleyi*, but there are no studies on the long-term effect of acute exposure to AMP. In *Prochloron* sp., however, both no effect and improved growth rate patterns were observed, but the improved growth does not seem to be related to the AMP dose due to the fluctuation between both effects over time at each dose. In terms of PS II photochemistry, the *Prochloron* sp. minimal fluoresce (F_0) increased in the presence of AMP from day 14. The increase in F_0 may be indicative of stress suggesting damage to the PS II light-harvesting

complex (Krause and Weis 1991) resulting in a less efficient transfer to the PS II reaction center and in the variability observed in the F_v/F_m values obtained. The effects of β-lactam antibiotics might not be related to the known mechanism of action in prokaryotes for *Prochloron* sp.; our results showed altered PS II performance. *Prochloron* sp. as a cyanobacterium was expected to be widely affected by a broad-spectrum antibiotic such as AMP, as described in freshwater cyanobacteria (Ando et al. 2007; Liu et al. 2012b; Wu et al. 2014; Dias et al. 2015; Wang et al. 2018). Accordingly, Prabaharan et al. (1994) observed that the marine cyanobacteria *Phormidium valderanum* is able to resist AMP by using this antibiotic as a nitrogen source in N-depleted medium. It appears that the antibiotic might be metabolized and used as an additional nitrogen source in both marine species studied, but in the case of *Prochloron* sp. PS II performance might be altered by AMP. Conversely, freshwater cyanobacteria, such as the *M. aeruginosa* (Ma12D) addressed in this study, have been so far sensitive to low concentrations of β-lactams and other antibiotics (Dias et al. 2015). *Microcystis aeruginosa* was the only strain to exhibit high growth inhibition at all doses studied. In our study, no correlation was observed between growth inhibition and AMP concentration increase because the test concentrations were orders of magnitude higher than the described EC_{50} values (on the order of 5–10 μg L^{-1} at 7 to 13 days of toxicity) (Holten Lützhøft et al. 1999; Liu et al. 2012b; Dias et al. 2015). AMP sensitivity is justified because *M. aeruginosa* has a bacterial structure and a prokaryotic chloroplast nature (González-Pleiter et al. 2013), although it has a special complex cell wall structure (Graham et al. 2009). We observed that the inhibition effect decreased over time, suggesting the potential for increasing tolerance to AMP within the bioassay tubes, most likely related to the biodegradation of the antibiotic. Furthermore, extracellular MC-LR production was stimulated by AMP at high concentrations (14 mg L^{-1}) compared with the control. Our results are consistent with previous observations, in which both intracellular and extracellular MC content increased when exposed to AMP and antibiotic mixtures (Liu et al. 2012b, 2014; Wang et al. 2018). Some studies suggest that the stimulated release of MCs is due to the lysis of the cell wall or increased permeability (Liu et al. 2012b). However, the increasing trend over time also suggests an increased synthesis of MCs in response to AMP stress.

Differences in sensitivity displayed by the microalgae species towards AMP may be attributed to differences in the mechanisms of antibiotic resistance, including degradation, inactivation, or algae metabolization. β-lactamases are the enzymes capable of breaking and inactivating the β-lactam antibiotics that have been widely described in β-lactam-resistant Gram-negative organisms, both bacteria and cyanobacteria (Kushner and Breuil 1977; Prabaharan et al. 1994; Baquero et al. 2008). Regardless of the removal mechanism, the microalgae-mediated elimination of antibiotics might be relevant for environmental risk assessment. Likewise, these degradative profiles displayed by all the species studied might have applications beyond risk assessment. In pursuit of remediation and environmental monitoring, the potential of microalgae is evident in a broad number of publications in this regard (Arribas and Herrero-Payo 1979; Wilde and Benemann 1993; Watanabe 2001; Podola et al. 2004; Peña-Vázquez et al. 2009; Orellana et al. 2010; Ben Chekroun et al. 2014). Our results show that all five species, even the cyanobacterium *M. aeruginosa*, were able to accomplish AMP biodegradation with high efficiency. Complete removal of AMP occurred within 14 days in four of the strains. There are apparently few reports on the biodegradation of ampicillin by microalgae (Prabaharan et al. 1994), but research on the capacity of microalgae to degrade antibiotics can be considered a promising prospect.

Conclusion

In summary, the present study explored the effect of high concentrations of AMP on a battery of microalgal and cyanobacterial species, assessing the responses over a month. The results revealed that the species susceptibility to this antibiotic varied greatly; no effect was observed in the chlorophytes, *M. aeruginosa* presented high inhibition and MC stimulation, upregulation/enhancement of *E. huxleyi* was observed, and photochemical stress was reported for the marine cyanobacteria *Prochloron* sp. These patterns of response indicate that eukaryote microalgae species are naturally nonsusceptible and even thrive in the presence of AMP and that cyanobacteria are affected in different ways not always directly related to the known mechanism of action of β-lactams, suggesting a significant role of AMP in the ecology and dynamics of phytoplankton communities. On this basis, we suggest that each aquatic ecosystem should be addressed specifically, considering the species variability and variation in AMP concentrations. Moreover, the results strongly suggest that biotic factors are implicated in the observed high rate of AMP degradation. Our results offer insight into the use of microalgae in bioremediation practices that could be applied in wastewater treatment plants (WWTPs).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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